



PCT/GB 2004 / 0 0 2 5 8 9

G 804 12589



INVESTOR IN PEOPLE

**PRIORITY DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH  
RULE 17.1(a) OR (b)

The Patent Office  
Concept House  
Cardiff Road  
Newport  
South Wales  
NP10 800

REC'D 13 JUL 2004	
WIPO	PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

*Andrew Gersey*

Dated 1 July 2004



1/77  
17JUN03 08:55:37 1 D01886  
P01/7700 0.00-0313995.3

# Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road  
Newport  
South Wales  
NP10 8QQ

1. Your reference

RD-ATC-33

2. Patent application number

(The Patent Office will fill in this part)

0313995.3

17 JUN 2003

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Advanced Technologies (Cambridge) Limited  
Globe House  
1 Water Street  
London WC2R 3LA

Patents ADP number (if you know it)

~~57476442002~~

75 773 56002

If the applicant is a corporate body, give the country/state of its incorporation

England & Wales

4. Title of the invention

Plant Limit Dextrinase Inhibitor Promoter

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Margot Ruth WALFORD  
Patents Department  
British American Tobacco  
R&D Centre  
Regents Park Road  
Southampton SO15 8TL

Patents ADP number (if you know it)

07515778004

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

YES

- a) any applicant named in part 3 is not an inventor, or
  - b) there is an inventor who is not named as an applicant, or
  - c) any named applicant is a corporate body.
- See note (d))

## Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form

Description

19

Claim(s)

Abstract

Drawing(s)

21

+ 21

Sw

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

4

sequence listing

✓

11. I/We request the grant of a patent on the basis of this application.

Signature

*Mr Reiner Diederich*

Date 16.6.83

12. Name and daytime telephone number of person to contact in the United Kingdom

Mr Reiner Diederich

02380 793731

### Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

### Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.
- Write your answers in capital letters using black ink or you may type them.
- If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- Once you have filled in the form you must remember to sign and date it.
- For details of the fee and ways to pay please contact the Patent Office.

## LIMIT DEXTRINASE INHIBITOR PROMOTER

### 1. FIELD OF INVENTION

The invention relates generally to a DNA sequence identified from *Hordeum vulgare* which acts as a tissue specific promoter. The invention further relates to the use of said DNA sequence to regulate exogenous gene expression in the endosperm and aleurone tissues of the seed of a plant.

### 2. BACKGROUND

The expression of a gene is dependent upon its DNA sequence being transcribed into RNA by the action of RNA polymerase. To achieve this, the RNA polymerase must recognise and attach to a region of DNA sequence located upstream of (i.e. 5' to) the gene coding sequence in order for transcription to be initiated. Such a region is termed the promoter of the gene. The intrinsic nature of the promoter sequence determines the circumstances and the manner in which the gene is expressed.

There are, broadly speaking, four types of promoters found in plant tissues; constitutive, tissue-specific, developmentally-regulated, and inducible/repressible.

A constitutive promoter directs the expression of a gene throughout the various parts of a plant continuously during plant development, although the gene may not be expressed at the same level in all cell types. Examples of known constitutive promoters include those associated with the cauliflower mosaic virus 35S transcript (Odell et al, 1985, Nature 313 810-812), the rice actin 1 gene (Zhang et al, 1991, Plant Cell 3 1155-1165) and the maize ubiquitin 1 gene (Cornejo et al, 1993, Plant Molec. Biol. 29 637-646).

A tissue-specific promoter is one which directs the expression of a gene in one (or a few) parts of a plant, usually throughout the lifetime of those plant parts. The category of tissue-specific promoter commonly also includes tissue-preferred or tissue-enhanced promoters which may also direct expression at some level in tissues other than the preferred tissue. Examples of tissue-specific promoters known in the art include those associated with the patatin gene expressed in potato tuber and the high molecular weight glutenin gene expressed in wheat, barley or maize endosperm.

A developmentally-regulated promoter directs a change in the expression of a gene in one or more parts of a plant at a specific time during plant development. The gene may be expressed in that plant part at other times during plant development at a different (usually lower) level, and may also be expressed in other plant parts.

An inducible promoter is capable of directing the expression of a gene in response to an inducer. In the absence of the inducer the gene will not be expressed. The inducer may act directly upon the promoter sequence, or may act by counteracting the effect of a repressor molecule. The inducer may be a chemical agent such as a metabolite, a protein, a growth regulator, or a toxic element, a physiological stress such as heat, wounding, or osmotic pressure, or an indirect consequence of the action of a pathogen or pest. A developmentally-regulated promoter might be described as a specific type of inducible promoter responding to an endogenous inducer produced by the plant or to an environmental stimulus at a particular point in the life cycle of the plant. Examples of known inducible promoters include those associated with wound response, such as described by Warner et al (1993), temperature response as disclosed by Benfey & Chua (1989), and chemically induced, as described by Gatz (1995).

A promoter sequence may comprise of a number of defined domains necessary for its function. The first of these defined domains comprises approximately 70 base pairs located immediately upstream of (5' to) the structural gene and forms the core promoter. The core promoter contains the CAAT and TATA boxes and defines the transcription initiation site for the structural gene. A series of regulatory sequences upstream of the core promoter constitute the remainder of the promoter sequence and determine the expression levels, the spatial and temporal patterns of expression, and the response to inducers. In addition some promoters contain sequence elements which act to enhance the level of expression, for example that from the pea plastocyanin promoter as described in International Patent Application, Publication No. WO 97/20056.

Genetic modification of plants depends upon the introduction of chimaeric genes into plant cells and their controlled expression under the direction of a promoter. Promoters may be obtained from different sources including plants, fungi, bacteria and viruses, and different promoters may work with different efficiencies in different tissues.

It may often be desirable to express introduced genes in a number of different tissues within a plant. For example, the expression of a resistance to a pathogen or pest, or tolerance to temperature extremes might be best expressed throughout all tissues in the plant. Similarly it might be desirable to ensure the expression of the transgenes at all times throughout the development of the plant. Also, a promoter which is expressed in a manner that is immune to the influence of inducers or repressors resulting from unforeseen environmental stimuli may also be useful to ensure the continued expression of a trait. For these purposes, the use of a "constitutive" promoter would be desirable. Examples of constitutive promoters include the

CaMv 35S promoter. For cereals the ubiquitin promoter is a constitutive promoter of choice (Christensen & Quail, 1996)

However, in some instances it is more desirable to control the location of gene expression in a transgenic plant to enhance the effect of gene expression by ensuring that expression occurs preferentially in those tissues where the effect of the gene product is most efficacious. By the same argument, modulated expression can reduce potential yield loss by reducing the resource drain on the plant. Further advantages include limiting the expression of agronomically useful yet generally deleterious genes to specific tissues by localisation and compartmentalisation of gene expression in cases where the gene product must be restricted to, or excluded from, certain tissues. For example, anther specific expression of the *suc* inhibitor genes (Mariani et al, 1990, Nature 347 737) has been used in male sterility systems, whereas expression in other parts of the plant would result in toxicity.

Examples of endosperm specific barley promoters include the high molecular weight glutenin promoter (Halford et al ). However, there is a lack of suitable endosperm specific promoters.

### 3. SUMMARY OF THE INVENTION

The present invention is directed to a DNA sequence identified from *Hordeum vulgare* (barley).

The present invention provides a DNA sequence comprising a promoter sequence, the DNA sequence being selected from the group consisting of: (a) a DNA sequence, the sequence being known herein as SEQ. ID. No:1; (b) a DNA sequence comprising a portion of the sequence of the isolated DNA of (a), and being capable of regulating the expression of a gene; (c) a DNA sequence, which sequence is at least 95% , 90%, 85% 80%, 75% or 70% homologous to the DNA sequence of (a) or (b) above; (d) a DNA sequence, which sequence is at least 80% identical to the DNA sequence of (a) or (b) and which is capable of regulating the expression of a gene; (e) a DNA sequence comprising a portion of the sequences of the DNAs of any one of (a) - (d) above and being capable of regulating the expression of a gene; (f) a DNA sequence, which sequence is complementary to the DNA sequence of any one of (a) to (e) above.

Preferably the DNA sequence is capable of regulating expression of a gene which encodes a limit dextrinase inhibitor protein (LDI).

More preferably the DNA sequence regulates expression of the gene encoding the Limit dextrinase inhibitor-like protein in *Hordeum vulgare*. Most preferably the DNA sequence regulates expression of the gene encoding the Limit dextrinase inhibitor protein in the endosperm or aleurone tissues of developing seeds of *Hordeum vulgare*.

The DNA sequences referred to herein may be isolated DNA sequences or, alternatively, may be synthesised DNA sequences.

The present invention also provides a recombinant DNA comprising vector DNA and a DNA sequence as described in any one of (a) to (f) above. The recombinant DNA may suitably further comprise the DNA coding sequence of a gene.

Preferably the recombinant DNA resides in a host cell suitable for transcription and translation.

The present invention also provides a method of regulating the expression of a gene, the method utilising the DNA sequence as described in any one of (a) to (f) above operably associated with the coding sequence of a gene.

The present invention also provides a transgenic plant, the cells of which plant comprise the DNA as described in any one of (a) to (f) above operably associated with a gene coding sequence.

The present invention further provides a method of modifying the metabolism within the cells of a transgenic plant utilising the DNA sequence as described in any one of (a) to (f) above operably associated with the coding sequence of a gene.

The present invention even further provides a method of producing a gene product within the cells of a transgenic plant utilising the DNA sequence as described in any one of (a) to (f) above operably associated with the coding sequence of a gene.

The present invention also provides an oligonucleotide probe which selectively hybridizes to a DNA sequence as described in any one of (a) to (f) above.

### 3.1 SEQUENCE IDENTIFIERS

In the sequence listing:

SEQ ID No. 1 shows the DNA sequence of the isolated barley limit dextrinase inhibitor promoter.

SEQ ID No. 2 shows the nucleotide and derived amino acid sequence of the isolated barley limit dextrinase inhibitor protein.

SEQ ID No. 3 shows the derived amino acid sequence of the isolated barley limit dextrinase inhibitor protein.

SEQ ID No. 4 shows the nucleotide sequence of the PCR primer inhib5.

SEQ ID No. 5 shows the nucleotide sequence of the PCR primer inhib6.

SEQ ID No. 6 shows the nucleotide sequence of the PCR primer AP1.

SEQ ID No. 7 shows the nucleotide sequence of the PCR primer GSP1.

SEQ ID No. 8 shows the nucleotide sequence of the PCR primer AP2

SEQ ID No. 9 shows the nucleotide sequence of the PCR primer GW1.

#### 4. BRIEF DESCRIPTION OF THE FIGURES

In order that the invention may be readily carried into effect reference will now be made, by way of example, to the following diagrammatic drawings in which;

Figure 1 shows the results of an RT-PCR analysis of barley limit dextrinase inhibitor expression. The RT-PCR analysis of LDI expression was performed using Inhib 5 and Inhib 6 primers.

Legend:  $\lambda$ /Hind III molecular weight markers ( $\lambda$ ); complete barley grains 20 dpa (1); complete barley grains 40 dpa (2); endosperm of barley grains 14 dpa (3); aleurone layers of barley grains treated with gibberellic acid (4); negative control of RT-PCR (5); days post anthesis (dpa);

Figure 2 shows the results of Northern blot analysis of limit dextrinase expression. The top panel of the blot shows the RNA gel and the bottom panel of the blot shows the Northern blot. Northern blot analysis of LDI expression was performed using LDI RNA probe on different RNA samples. Legend: Leaves in the light (LL); leaves in the dark (LD); roots (r); post anthesis (p.a.);

Figure 3 shows the results of Southern blot analysis of limit dextrinase inhibitor in barley genomic DNA. The Southern analysis of barley ("Golden Promise") DNA was performed using limit dextrinase inhibitor DNA as a probe. Legend:  $\lambda$ /Hind III molecular weight markers ( $\lambda$ ); DNA digested with EcoR V (E); DNA digested with BamH1 (B); DNA digested with Xho I (X);

Figure 4 shows an isolation of limit dextrinase inhibitor promoter fragments by genome walking PCR. DNA was digested with DraI, SmaI, EcoR V, Pvu II and Sca I. The primers used were AP2 and GW 1, and the PCR products analyzed on a preparative 1% TAE agarose gel. Legend:  $\lambda$ /Hind III molecular weight markers ( $\lambda$ ); negative control of PCR reaction (-ve). Base pairs (bp);

Figure 5 shows a Barley transformation construct containing the limit dextrinase inhibitor promoter pCAMBIA1302 with LDI promoter;



Figure 6 shows a transient expression assay using the limit dextrinase promoter: GFP construct. Fluorescent microscopy was performed on endosperm 2-4 weeks post anthesis transiently assayed with an LDI Promoter: GFP construct. Legend: Non-bombarded controls (A, C, E and G); bombarded with LDI Promoter:GFP construct and analyzed for GFP expression 24h and 48h after bombardment (B, D, F and H). Scaling bar is 100µm in length.

Figure 7 shows the putative transcription start sites and TATA sequences (-25 bp) within SEQ. ID. No: 1 as predicted by neural network (M.G. Reese, N.L. Harris & F.H. Eeckman. 1996; Large Scale Sequencing Specific Neural Network for promoter & slice site recognition: Biocomputing Proceedings of 1996 Pacific Symposium). The putative transcript start site is shown in larger font.

Figure 8 shows the results of a full sequence search using the BLASTN algorithm for sequences similar to SEQ. ID. No. 1 of the present invention. The search results provided no significant homology with any known sequence in the four databases searched.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to plant gene regulatory sequences. Specifically this invention relates to a promoter identified in *Hordeum vulgare* (barley).

A plant DNA sequence may be recovered from the cells of the natural host or it may be synthesized directly *in vitro*. Extraction from the natural host enables the isolation *de novo* of novel sequences, whereas *in vitro* DNA synthesis generally requires pre-existing sequence information. Direct chemical *in vitro* synthesis can be achieved by sequential manual synthesis or by automated procedures. DNA sequences may also be constructed by standard techniques of annealing and ligating fragments or by other methods known in the art. Examples of such cloning procedures are given in Sambrook et al.

The DNA sequence of the present invention may be isolated by direct cloning of segments of plant genomic DNA and screening the DNA sequence for the presence of diagnostic sequence motifs characteristic of known promoter sequences. Suitable segments of genomic DNA may be obtained by fragmentation using restriction endonucleases, sonication, physical shearing, or other methods known in the art.

The identification of the cloned segment as a promoter sequence may alternatively be achieved by assessing functionality, for example by linking the cloned segment with a coding sequence derived from a reporter gene and introducing the chimaeric construct into a host cell or cell-free system wherein expression of the reporter gene can be evaluated. This process may form part of another sequence isolation strategy termed promoter trapping, wherein genomic

DNA fragments are cloned directly into "expression vectors" comprising a reporter gene coding region and other sequences necessary for expression in a host cell or cell-free system. The expression may or may not require integration of the chimaeric construct into the host's chromosomal DNA (Topping J F. *et al.* (1991) and Topping J F. *et al.* (1994)).

An alternative method of obtaining DNA sequences of the present invention is by the identification and isolation of a DNA coding sequence which is known to be expressed and subsequently using this sequence to obtain the contiguous promoter sequence, which is by definition directing the expression of the coding sequence. A coding sequence may be obtained by the isolation of messenger RNA (mRNA or polyA+ RNA) from plant tissue. The tissue used for RNA isolation is selected on the basis that suitable gene coding sequences are believed to be expressed in that tissue at optimal levels for isolation.

Various methods for isolating mRNA from plant tissue are well known to those skilled in the art, including for example using an oligo-dT oligonucleotide immobilised on an inert matrix. The isolated mRNA may be used to produce its complementary DNA sequence (cDNA) by use of the enzyme reverse transcriptase (RT) or other enzymes having reverse transcriptase activity. Isolation of an individual cDNA sequence from a pool of cDNAs may be achieved by cloning into bacterial or viral vectors, or by employing the polymerase chain reaction (PCR) with selected oligonucleotide primers. The production and isolation of a specific cDNA from mRNA may be achieved by a combination of the reverse transcription and PCR steps in a process known as RT-PCR.

In order to isolate the promoter sequence of the present invention, a cDNA sequence to a Limit dextrinase inhibitor protein may be identified. Examples of other Limit dextrinase inhibitor protein genes are given in Genbank Accession No.s X13443 for the *Hordeum vulgare* alpha-amylase/trypsin inhibitor; X99982. for the *Triticum aestivum* cDNA for the PUP88 protein ; AJ222975 for the *Hordeum spontaneum* cDNA for the Itr1 gene for BTI-Cme2.2 protein; AP005197 for the *Oryza sativa* cDNA for the putative hageman factor inhibitor protein ; X61032 for the *Triticum durum* cDNA for the alpha amylase inhibitor protein; and X54064 for the *Zea mays* cDNA for the Hageman factor inhibitor protein .

Limit dextrinase inhibitor proteins suitable for use in isolating the promoter of the present invention include proteins having the amino acid sequence given as SEQ. ID. No:3 and proteins homologous to, and having essentially the same biological properties as, the protein disclosed herein as SEQ. ID. No. 3. This definition is intended to encompass natural allelic variations in the protein plus any non-allelic examples. It will be appreciated that the amino acid sequence need not be identical to that of SEQ. ID. No. 3; for the purposes of this invention, the amino acid

sequence may be at least 80%, 90%, or 95% homologous or more with the protein of SEQ. ID. No:3 to retain its biological activity. General categories of potentially equivalent amino acids include, but are not limited to: glutamic acid and aspartic acid; lysine, arginine and histidine; alanine, valine, leucine and isoleucine; asparagine and glutamine; threonine and serine; phenylalanine, tyrosine and tryptophan; and glycine and alanine.

The selected cDNA may then be used to evaluate the genomic features of its gene of origin by use as a hybridisation probe in a Southern blot of plant genomic DNA to reveal the complexity of the genome with respect to that sequence. Alternatively, sequence information from the cDNA may be used to devise oligonucleotides and these may be used in the same way as hybridisation probes for PCR primers to produce hybridisation probes or for PCR primers to be used in direct genome analysis.

Similarly the selected cDNA may be used to evaluate the expression profile of its gene of origin by use as a hybridisation probe in a Northern blot of RNA extracted from various plant tissues, or from a developmental or temporal series. Again sequence information from the cDNA may be used to devise oligonucleotides which can be used as hybridisation probes to produce hybridisation probes or directly for RT-PCR.

The selected cDNA, or derived oligonucleotides, may then be used as a hybridisation probe to challenge a library of cloned genomic DNA fragments and identify overlapping DNA sequences. By this means a contiguous promoter may be identified and isolated.

By the nature of the method of isolation, an isolated cDNA usually comprises the 3' terminus of the coding region and extends towards the 5' terminus. It may not comprise the full-length coding sequence. It is preferable to ensure that the 5' terminal sequence is present if the cDNA is to be used to identify the contiguous promoter. This may be achieved by extension of the cloned cDNA sequence in the 5' direction by a process termed 5' RACE.

If sequence analysis of the cloned cDNA identifies a homologous sequence already reported in the scientific literature, this information may provide a suitable candidate sequence for the 5' terminus. However the possibility of there being different members of the same gene family with similar coding regions, but differing intron regions, promoter sequences and expression profiles may lead to the selection of an incorrect and unsuitable promoter sequence.

Once the 5' terminus of the coding sequence has been identified, the contiguous upstream region containing the promoter may be isolated by further extension in the 5' direction. This may be achieved by methods including vector-ligation PCR, genome walking, vectorette PCR, and other methods. If necessary the process may be repeated with a new primer complementary

to the 5' terminus of the first promoter fragment to ensure that all the control sequences of the promoters are isolated.

The present invention provides a DNA sequence which controls the expression of a Limit dextrinase inhibitor protein which DNA sequence is known herein as SEQ. ID. No. 1, and which may be used as a plant promoter. The present invention also includes within its scope DNA sequences which control the expression of other Limit dextrinase inhibitor protein genes and which are homologous with the DNA sequence of SEQ. ID. No. 1.

Homology may be determined on the basis of percentage identity between two DNA (or polypeptide) sequences. "Percentage identity", as known in the art, is a measure of the relationship between two polynucleotides or two polypeptides, as determined by comparing their sequences. In general the two sequences to be compared are aligned to give a maximum correlation between the sequences. The alignment of the two sequences is examined and the number of positions giving an exact nucleotide (or amino acid) correspondence between the two sequences determined, divided by the total length of the alignment multiplied by 100 to give a percentage identity figure. This percentage identity figure may be determined over the whole length of the sequences to be compared, which is particularly suitable for sequences of the same or very similar lengths and which are highly homologous, or over shorter defined lengths, which is more suitable for sequences of unequal length or which have a lower level of homology.

For example, sequences can be aligned with the software clustalw under Unix which generates a file with the ".aln" extension, this file can then be imported into the Bioedit program (Hall, T.A. 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98) which opens the .aln file. In the Bioedit window, one can choose individual sequences (two at a time) and align them. This method allows for comparison of entire sequences.

Methods for comparing the identity of two or more sequences are well known in the art. Thus for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux J. et al, Nucleic Acids Res. 12 387-395, 1984, available from Genetics Computer Group, Madison, Wisconsin, USA), for example the programs BESTFIT and GAP, may be used to determine the percentage identity between two polynucleotides and the percentage identity between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman (Advances in Applied Mathematics, 2 482-489, 1981) and finds the best single region of similarity between two sequences. BESTFIT is more suited to comparing two polynucleotide or two polypeptide sequences which are dissimilar in length, the program assuming that the shorter sequence represents a portion of the longer. In comparison, GAP

aligns two sequences finding a "maximum similarity" according to the algorithm of Needleman and Wunsch (J. Molec. Biol. 48 443-454, 1970). GAP is more suited to comparing sequences which are approximately the same length and an alignment is expected over the entire length. Preferably the parameters "Gap Weight" and "Length Weight" used in each program are 50 and 3 for polynucleotide sequences and 12 and 4 for polypeptide sequences, respectively. Preferably, percentage identities and similarities are determined when the two sequences being compared are optimally aligned.

Other programs for determining identity and/or similarity between sequences are also known in the art, for instance the BLAST family of programs (Karlin & Altschul, 1990, *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin & Altschul, 1993, *Proc. Natl. Acad. Sci. USA* 90:5873-5877, available from the National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA and accessible through the home page of the NCBI at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). These programs exemplify a preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al., 1990, *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997, *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, *CABIOS* 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

Another non-limiting example of a program for determining identity and/or similarity between sequences known in the art is FASTA (Pearson W.R. and Lipman D.J., *Proc. Nat. Acad. Sci., USA*, 85:2444-2448, 1988, available as part of the Wisconsin Sequence Analysis Package). Preferably the BLOSUM62 amino acid substitution matrix (Henikoff S. and Henikoff J.G., *Proc.*

Nat. Acad. Sci., USA, 89:10915-10919, 1992) is used in polypeptide sequence comparisons including where nucleotide sequences are first translated into amino acid sequences before comparison.

Yet another non-limiting example of a program known in the art for determining identity and/or similarity between amino acid sequences is SeqWeb Software (a web-based interface to the GCG Wisconsin Package: Gap program) which is utilized with the default algorithm and parameter settings of the program: blosum62, gap weight 8, length weight 2.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

Preferably the program BESTFIT is used to determine the % identity of a query polynucleotide or a polypeptide sequence with respect to a polynucleotide or a polypeptide sequence of the present invention, the query and the reference sequence being optimally aligned and the parameters of the program set at the default value.

In the context of the present invention "substantially homologous" sequences are those which have at least 45% sequence identity, preferably at least 50% sequence identity, more preferably at least 60%, 70%, 80%, or 90% sequence identity and most preferably at least 95% sequence identity with the described sequences. In some cases sequence identity may be 98% or more preferably 99%, or more.

The present invention also includes DNA sequences which hybridize to the above isolated DNA, including partial sequences and complementary sequences. Conditions under which such sequences will so hybridize can be determined in a routine manner. For example, the present invention includes within its scope, DNA sequences which hybridize to the DNA sequences of the present invention under stringent conditions. In the context of the present invention, "stringent conditions" are defined as those given in Martin *et al* (EMBO J 4:1625-1630 (1985)) and Davies *et al* (Methods in Molecular Biology Vol 28: Protocols for nucleic acid analysis by non-radioactive probes, Isaac, P.G. (ed) pp 9-15, Humana Press Inc., Totowa N.J, USA)). The conditions under which hybridization and/or washing can be carried out can range from 42°C to 68°C and the washing buffer can comprise from 0.1 x SSC, 0.5 % SDS to 6 x SSC, 0.5 % SDS. Typically, hybridization can be carried out overnight at 65°C (high stringency conditions), 60°C (medium stringency conditions), or 55°C (low stringency conditions). The filters can be washed for 2 x 15 minutes with 0.1 x SSC, 0.5 % SDS at 65°C (high stringency washing). The filters were washed for 2 x 15 minutes with 0.1 x SSC, 0.5 %

SDS at 63°C (medium stringency washing). For low stringency washing, the filters were washed at 60°C for 2 x 15 minutes at 2 x SSC, 0.5% SDS.

The present invention also includes DNA sequences which hybridize to oligonucleotide probes. Preferably the DNA sequences hybridize to oligonucleotide probes under stringent conditions. In instances wherein the nucleic acid molecules are oligonucleotides ("oligos"), highly stringent conditions may refer, for example, to washing in 6x SSC / 0.05% sodium pyrophosphate at 37°C (for 14 base oligos), 48°C (for 17 base oligos), 55°C (for 20 base oligos), and 60°C (for 23 base oligos).

The gene coding sequence employed in carrying out the present invention may be active in some or all plant tissues. The sequence employed may encode a protein or an RNA moiety. Through recombinant DNA techniques the sequence may encode a synthetic variant of a protein or RNA, a partial sequence, or a composite sequence comprising regions from one or more genes. For example, the chimaeric gene may encode a polyprotein. The sequence may also comprise repeated, inverted or complementary sequences, to achieve disruption of the transcription and translation of an endogenous gene or genes by, for example, antisense or RNA inhibition technology.

The sequence known herein as SEQ. ID. No. 2 includes a coding sequence therein for the gene encoding a limit dextrinase inhibitor protein. In addition, many plant, bacterial and viral genes may be actively expressed. Exemplary genes encode the GUS, GFP and luciferase enzymes which may be used as reporter genes for promoter function. In addition the LDI promoter may be used to direct the expression of DNA sequences designed to alter the metabolism of the endosperm of the seeds of cereal plants, especially barley. Such genes include those for carbohydrate metabolism, starch metabolism, amino acid and protein metabolism and lipid metabolism.

The gene product of the present invention can be produced by recombinant techniques, wherein genomic DNA clones or cDNA clones for the DNA coding sequence are produced, isolated, proliferated, and incorporated into a plant transformation vector of the present invention.

### **Plant transformation vectors**

Plant transformation vectors of the present invention will contain "expression cassettes" comprising 5'-3' in the direction of transcription, a promoter sequence as described in the present invention, a gene coding sequence as discussed above and, optionally a 3' untranslated,

terminator sequence including a stop signal for RNA polymerase and a polyadenylation signal for polyadenylase.

The promoter sequence may be present in one or more copies, and such copies may be identical or variants of the promoter sequence as described above. Such copies may also be complete or partial sequences as described above.

The terminator sequence may be obtained from plant, bacterial or viral genes. Suitable terminator sequences are the pea *rbcS* E9 terminator sequence, the *nos* terminator sequence derived from the nopaline synthase gene of *Agrobacterium tumefaciens* and the 35S terminator sequence from cauliflower mosaic virus, for example. A person skilled in the art will be readily aware of other suitable terminator sequences.

The expression cassette may also comprise a gene expression enhancing mechanism to increase the strength of the promoter. An example of such an enhancer element is that derived from a portion of the promoter of the pea plastocyanin gene, and which is the subject of International Patent Application, Publication No. WO 97/20056.

These regulatory regions may be derived from the same gene as the promoter DNA sequence of the present invention or may be derived from different genes, e.g. from *Hordeum vulgare* or other organisms. All of the regulatory regions should be capable of operating in cells of the tissue to be transformed.

The gene coding sequence may be derived from the same gene as the promoter DNA sequence of the present invention or may be derived from a different gene, e.g. from *Hordeum vulgare* or another organism.

The expression cassette may be incorporated into a basic plant transformation vector, such as, binary or super-binary vectors, for example, pBIN19 and pBIN+ and other suitable plant transformation vectors known in the art.

In addition to the expression cassette, the plant transformation vector will contain such sequences as are necessary for the transformation process. These may include the *Agrobacterium vir* genes, one or more T-DNA border sequences, and a selectable marker or other means of identifying transgenic plant cells.

### **Production of the gene product**

Expression of a DNA coding sequence in the plant host cell will produce an RNA transcript. If the coding sequence is derived from a structural gene, the RNA transcript is then translated into a protein gene product. If desired, the gene product can be isolated by standard techniques for isolating proteins from biological systems, such as salt precipitation, column



chromatography, immunoaffinity techniques, electrophoresis, recrystallisation, centrifugation, and such like.

### **Example 1. Isolation of barley *limit dextrinase inhibitor* cDNA fragments.**

#### *Cloning of LDI gene by RT-PCR*

Total RNA was extracted from barley (var. Golden Promise) grains 2 and 4 weeks post anthesis with a LiCl method as described by (Cathala *et al.*, 1983).

2 µg of RNA was treated with Rnase-free DNase I (Amersham Pharmacia Biotech) and used to synthesize 30 µl first strand cDNA using random hexamer primers (Roche) and M-MLV reverse transcriptase (Promega) using the reaction conditions recommended by the manufacturer.

It will be recognised by one skilled in the art that other mRNA extraction and cDNA synthesis methods exist which could be employed to produce cDNA from tissue of *Hordeum vulgare*.

A 3 µl aliquot of the cDNA product was used in a standard PCR reaction containing 2 mM MgCl<sub>2</sub>, 8% (v/v) DMSO and primers Inhib-5 (5'-ACCAATAAACTAGTATCAACAATGGCATCCGACCA-3' SEQ ID No 4) and Inhib-6 (5'-CCAACCTTTTTTATTCATCAATCGGCCACA-3' SEQ ID No 5), which were designed against the *Hordeum vulgare* Limit dextrinase inhibitor sequence (Genbank Accession no. X13443.), using Taq polymerase (Bioline) as recommended by the manufacturer. The PCR program used was 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 sec, 63.5 °C for 30 sec and 72 °C for 1 min, finalized by 7 min at 72 °C. The product length was 623 bp. The amplified product was cloned into a pGEM-T Easy vector (Promega) and verified by sequencing.

It will be recognised by one skilled in the art that other methods exist which could be employed to produce cloned DNA fragments.

The sequence of this cDNA clone is shown in SEQ ID No 2. Compared to the published sequence (Genbank Accession no. X13443.), there are five single base pair substitutions, which in turn lead to two amino acid substitutions.

### **Example 2. RT-PCR analysis of Limit Dextrinase Inhibitor expression.**

Total RNA was isolated from barley tissues using a LiCl method (M.-J.Cho *et al.*, 1998). 2 µg of total DNase treated RNA was used to produce cDNA with M-MLV reverse transcriptase. 1/10 of this cDNA was used to run a PCR reaction with specific primers for the entire coding region of LDI.

The expression of the LDI gene was investigated by using RT-PCR with the specific primers Inhib 5 (SEQ ID No. 4) and Inhib 6 (SEQ ID No. 5) on RNA from developing grains. Expression of LDI was found in grains 20 and 40 days post anthesis, the endosperm and gibberellic acid treated aleurone layers. The highest level of expression was found in the endosperm. As shown in Figure 1.

**Example 3. Northern analysis of Limit Dextrinase Inhibitor expression.**

Total RNA was isolated from developing and germinating barley grains, barley leaves in the light or dark, and barley roots using a LiCl method (M.-J.Cho et al., 1998). 5 µg of total RNA were separated on a 1 % agarose gel with formaldehyde. The RNA was blotted over night with 10× SSC onto a positively charged Nylon membrane and UV crosslinked. The blot was then prehybridized in DIG-EasyHyb and hybridised with 100 ng/ml DIG-labeled RNA probe at 68 °C over night. The blot was washed once in 2× SSC, followed by 2 washes with 0.2× SSC at 68 °C. After that the blot was blocked for 1 hour at RT in blocking reagent. This was followed by 30 minutes in 1:10,000 dilution of anti-DIG antibody solution. To remove excess antibody the blot was washed 4× in Tris buffer with salt, then equilibrated in phosphatase buffer and the chemiluminiscent substrate (CDP-star) added. Chemiluminescence detection using X-ray film was performed. Northern blotting and hybridisation was carried out as detailed by manufacturers of the DIG label (Roche).

Expression of LDI was only detected in early development of barley grains (2 and 4 weeks post anthesis). No expression was found in germinating grains and vegetative tissue as shown in Figure 2.

**Example 4. Southern blotting**

Genomic DNA of barley leaf tissue was isolated according to the method of A.W.MacGregor *et al.*, 1995 and S.L.Delaporta *et al.*, 1983. 10 µg digested genomic DNA (isolated using the Ambion Phytopure Kit according to manufacturer's instructions) was separated on a 0.8 % TAE agarose gel at 20V over night. The DNA was depurinated in 250 mM HCl for 10 minutes, denatured for 30 min in 0.4 M NaOH and neutralized before blotting for 8 hours onto a positively charged Nylon membrane (Roche) in 20× SSC. The DNA was UV crosslinked and prehybridized in DIG-Easyhyb for 1 hour at 52 °C and hybridised with 40ng/ml DIG labeled DNA probe at 52 °C over night. Washing and detection was carried out as described before for Northern blotting except for a more stringent wash with 0.1 x SSC.

Southern blot analysis was used to examine the number of gene copies in wildtype (wt) "Golden Promise" barley. The exact number of copies of LDI in wildtype "Golden Promise" barley is hard to estimate as several bands per digest are visible. The most likely explanation is that there is a small gene family of related genes present in barley, as shown in Figure 3.

**Example 5. Isolation of the limit dextrinase inhibitor promoter region by genome walking.**

Genomic DNA of barley leaf tissue was isolated according to the method of A.W.MacGregor et al., 1995 and S.L.Delaporta et al., 1983. 2.5 µg genomic barley DNA (variety "Golden Promise") was digested with the restriction enzymes Sca I, Pvu II, EcoR V, Sma I or Dra I respectively. These DNA fragments were then ligated to an asymmetric adapter and used as a template for PCR reactions with adapter primers and gene specific primers to isolate the 5' region of the LDI gene. All procedures were carried out as described in the Clontech manual. The first PCR was carried out using primers AP1 (SEQ ID No. 6 ) and GSP1 (SEQ ID No. 7 ). 1µl of this 50 µl PCR reaction was used as template for the secondary (nested) PCR using primers AP2 (SEQ ID No. 8 ) and GW1 (SEQ ID No. 9 ).

Using the procedures described above, two candidate fragments were isolated, from digests with Sma I and Pvu II, as shown in Figure 4. The DNA fragments from the Sma I (~1000 bp) and Pvu II (~ 650 bp) digests were purified and cloned into the Topo 4 vector and sequenced. Both fragments (Sma I and Pvu II) showed the same A and T rich sequence, spanning the 790 bp long 5' region of LDI. The promoter sequence is shown in SEQ ID No. 1. SEQ ID No. 1 discloses a nucleic acid sequence of 833 bp, within which sequence there is the 790 bp promoter sequence of the present invention. The sequence also contains therewithin the 5' untranslated region of the gene coding sequence, up to and including the first codon of the gene.

**Example 6. Transient Expression of Limit Dextrinase Inhibitor Promoter marker gene constructs.**

In order to investigate if the 790 bp region upstream of the LDI gene was sufficient as a promoter, a vector containing the putative promoter region plus a reporter gene was constructed. It consists of the 790 bp Sma I fragment, the GFP gene (green fluorescent protein) and a terminator (NOS). This construct (Figure 5) was used for transient assays on barley endosperm (2-4 weeks post anthesis) and for stable barley transformation. Endosperms were transformed with a biolistic device using construct DNA coated gold particles and analysed by fluorescence microscopy after 24 and 48 hours post bombardment. The results in shown in Figure 6

demonstrate the expression of GFP, showing that the isolated genomic sequence acts as promoter.

**Example 7. Stable expression of Limit Dextrinase Inhibitor Promoter marker gene constructs.**

Stable barley transformation of immature embryos of the barley variety "Golden Promise" was performed with a biolistic device using DNA coated gold particles. Selection and regeneration procedures were performed as described by Y.Wan & P.G.Lemaux, 1994.

## References.

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25** 3389-3402
- Ausubel, F.M., Bent, R., Kingston, R.E., Moore, D.D., Seidmann, J.G., Smith, J.A. & Struhl, K. (1989). *Current protocols in molecular biology*. New York: Greene and Willy Interscience.
- Benfey, P.N., and Chua, N-H. (1989) Regulated genes in transgenic plants. *Science* **244**, 174-181.
- Bevan, M. (1984) Binary *Agrobacterium* Vectors for Plant Transformation. *Nucleic Acids Research* **12** 8711-8721
- Buluwela, L., Forster, A., Boehm, T. & Rabbitts, T.H. (1989). A rapid procedure for colony screening using nylon filters. *Nucleic Acids Research* **17**, 452-452.
- Christensen, A. H. & Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* **5**, 213-218
- Cho, M.-J., Jiang, W. & Lemaux, P.G. (1998). Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. *Plant Science* **138**, 229-244.
- Conkling, M.A., Cheng, C-L., Yamamoto, Y.T. and Goodman, H.M. (1990) *Plant Physiol.* **93** 1203-1211
- Delaporta, S.L., Wood, J. R. & Hicks, J.B. (1983). A plant DNA miniprep: version 2. *Plant Molecular Biology Reporter* **1**, 19-22.
- Gatz, C. (1995) Novel inducible/repressible gene expression systems. *Methods in Cell Biol.* **50**, 411-424
- Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S.G. & Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science* **227**, 1229-1231.
- Jefferson, R.A. (1987) Assaying Chimeric Genes in Plants: The GUS Gene Fusion System. *Plant Molec. Biol. Repr.* **5** 387-405
- Kristensen, M., Planchot, V., Abe, J.-I. & Svensson, B (1998). Large-scale purification and characterization of barley limit dextrinase, a member of the  $\alpha$ -amylase structural family. *Cereal Chemistry* **75**, 473-479.
- Lazaro, A., Rodriguez-Palenzuela, P., Marana, C., Carbonero, P. & Garciaolmedo, F. (1988). Signal peptide homology between the sweet protein thaumatin II and unrelated cereal  $\alpha$ -amylase/trypsin inhibitors. *FEB* **239**, 147-150.

- Lemaux, P.G. Cho, M.-J., Zhang, S. & Bregitzer, P. (1999). Transgenic cereals: *Hordeum vulgare* L. (barley). In *Molecular improvement of cereal crops*, ed. I.K.VASIL, pp. 255-316. Dordrecht: Kluwer Academic Publishers.
- Macgregor, A.W., Macri, L.J., Bazin, S.L. & Sadler, G.W. (1995). Limit dextrinase inhibitor in barley and malt and its possible role in malting and brewing. *EBC Congress* 185-193.
- Macgregor, A.W., Macri, L.J., Schroeder, S.W. & Bazin, S.L. (1994). Purification and characterisation of limit dextrinase inhibitors from barley. *Journal of Cereal Science* 20, 33-41.
- Macgregor, E.A., Bazin, S.L., Ens, E.W., Lahnstein, J., Macri, L.J., Shirley N.J. & Macgregor, A.W. (2000). Structural models of limit dextrinase inhibitors from barley. *Journal of Cereal Science* 31, 79-90.
- Manners, D.J. (1997). Observations on the possible involvement of debranching enzymes in starch biosynthesis. *J.Appl.Glycosci.* 44, 541-546.
- Sambrook, J., Frisch, E.F. and Maniatis, T. (Eds.) (1989) *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> edn. Cold Spring Harbor Laboratory Press, New York.
- Topping J F; Wei W; Lindsey K; (1991) *Development* 112, 1009-1019. Functional tagging of regulatory elements in the plant genome
- Topping J F; Agyeman F; Henricot B; Lindsey K (1994) *The Plant Journal* 5, 895-903, Identification of molecular markers of embryogenesis in *Arabidopsis thaliana* by promoter trapping.)
- Wan, Y. & Lemaux, P.G. (1994). Generation of large numbers of independently transformed fertile barley plants. *Plant Physiology* 104, 37-48.

# SEQUENCE LISTING

<110> Advanced Technologies (Cambridge) Ltd.

<120> Limit Dextrinase Inhibitor Promoter

<130> RD-ATC-33

<140>

<141>

<160> 9

<170> PatentIn Ver. 2.1

<210> 1

<211> 833

<212> DNA

<213> Hordeum vulgare

<400> 1

```
catcgattac tatagggcac gcgtggcgca cggcccgggc tggtattgga caccaaatgt 60
atcataaact tgttttttca cgcacaaaat attgctcctc catttcgcat taaaattgtc 120
aagcatgctt gcaacagtaa cacgaacatt cataaaaaaa atatttttta agaaaacatt 180
tactattttt ttgttactat tcatctggga gcatgtgctt ccggaagcca aaatgcccct 240
tccaatatgc ccgtgtataa agaaacccct tctttcctaa aaatatatat catcgctcgt 300
catgatacgt ttatgtatcc aacgaaaaat attttcgcat gtcaccaaaa atgttttata 360
ttacacaagt gaacaaatat gataaactcc ctctgtgtaa ctattttttc tgtgaaataa 420
aaggatgaca atcaaaaaca aaatgtagac tgtaaaacaaa gaaaacatta tttcctagaa 480
ataaaaaaaaa agattagagg gatatgtatt gtcgaaacac atgaggacta gaacaaaaga 540
aaaagggaaa tgagaaggaa aaaaggggta accattaccc aaagaaaaca gaaagtaaac 600
tagacgtgtc gaagggaaac ggagtttgca ggggcgttcc aaattcagtt gcaagaacct 660
ccaaataaac gccacaaga aagaaatgag cattacttgc gcgctttgca ctcttatctc 720
tagcatctcc cgatacatat atacatgtag cctagctgca gatcttgaat agctattctt 780
gccaccagg ccaagagatt gaaccaacga ccaataaact agtatcaaca atg 833
```

<210> 2

<211> 517

<212> DNA

<213> Hordeum vulgare

<220>

<221> CDS

<222> (14) .. (457)

<400> 2

```
actagtatca aca atg gca tcc gac cat cgt cgc ttc gtc ctc tcc ggc 49
                Met Ala Ser Asp His Arg Arg Phe Val Leu Ser Gly
                  1                      5                      10

gcc gtc ttg ctc tcg gtc ctc gcc gtc gcc gcc gcc acc ctg gag agc 97
Ala Val Leu Leu Ser Val Leu Ala Val Ala Ala Ala Thr Leu Glu Ser
          15                      20                      25

gtc aag gac gag tgc caa cca ggg gtg gac ttc ccg cat aac ccg tta 145
Val Lys Asp Glu Cys Gln Pro Gly Val Asp Phe Pro His Asn Pro Leu
          30                      35                      40

gcc acc tgc cac acc tac gtg ata aaa cgg gtc tgc ggc cgc ggt ccc 193
Ala Thr Cys His Thr Tyr Val Ile Lys Arg Val Cys Gly Arg Gly Pro
          45                      50                      55                      60
```

agc cgg ccc atg ctg gtg aag gag cgg tgc tgc cgg gag ctg gcg gcc 241  
 Ser Arg Pro Met Leu Val Lys Glu Arg Cys Cys Arg Glu Leu Ala Ala  
                     65                    70                    75

gtc ccg gat cac tgc cgg tgc gag gcg ctg cgc atc ctc atg gac ggg 289  
 Val Pro Asp His Cys Arg Cys Glu Ala Leu Arg Ile Leu Met Asp Gly  
                     80                    85                    90

gtg cgc acg ccg gag ggc cgc gtg gtt gag gga cgg ctc ggt gac agg 337  
 Val Arg Thr Pro Glu Gly Arg Val Val Glu Gly Arg Leu Gly Asp Arg  
                     95                    100                    105

cgt gac tgc ccg agg gag gag cag agg gcg ttc gcc gcc acg ctt gtc 385  
 Arg Asp Cys Pro Arg Glu Glu Gln Arg Ala Phe Ala Ala Thr Leu Val  
                     110                    115                    120

acg gcg gcg gag tgc aac cta tgc tcc gtc cag gag ccg gga gta cgc 433  
 Thr Ala Ala Glu Cys Asn Leu Ser Ser Val Gln Glu Pro Gly Val Arg  
                     125                    130                    135                    140

ttg gtg cta ctg gca gat gga tga cgatcgaaat gcgccaaggt aatgaagcgg 487  
 Leu Val Leu Leu Ala Asp Gly  
                     145

agtactgtat acagaataaaa agtactcgag 517

<210> 3

<211> 147

<212> PRT

<213> Hordeum vulgare

<400> 3

Met Ala Ser Asp His Arg Arg Phe Val Leu Ser Gly Ala Val Leu Leu  
   1                    5                    10                    15  
 Ser Val Leu Ala Val Ala Ala Ala Thr Leu Glu Ser Val Lys Asp Glu  
                     20                    25                    30  
 Cys Gln Pro Gly Val Asp Phe Pro His Asn Pro Leu Ala Thr Cys His  
                     35                    40                    45  
 Thr Tyr Val Ile Lys Arg Val Cys Gly Arg Gly Pro Ser Arg Pro Met  
                     50                    55                    60  
 Leu Val Lys Glu Arg Cys Cys Arg Glu Leu Ala Ala Val Pro Asp His  
                     65                    70                    75                    80  
 Cys Arg Cys Glu Ala Leu Arg Ile Leu Met Asp Gly Val Arg Thr Pro  
                     85                    90                    95  
 Glu Gly Arg Val Val Glu Gly Arg Leu Gly Asp Arg Arg Asp Cys Pro  
                     100                    105                    110  
 Arg Glu Glu Gln Arg Ala Phe Ala Ala Thr Leu Val Thr Ala Ala Glu  
                     115                    120                    125  
 Cys Asn Leu Ser Ser Val Gln Glu Pro Gly Val Arg Leu Val Leu Leu  
                     130                    135                    140  
 Ala Asp Gly  
 145

<210> 4

<211> 35

<212> DNA

<213> Artificial Sequence



<220>

<223> Description of Artificial Sequence: PCR primer

<400> 4

accaataaac tagtatcaac aatggcatcc gacca

35

<210> 5

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer

<400> 5

ccaacctttt ttattcatca atcgccac

29

<210> 6

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR Primer

<400> 6

gtaatagcgc tcactatagg gc

22

<210> 7

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer

<400> 7

agacccgttt tatcacgtag gtgtgg

26

<210> 8

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer

<400> 8

actatagggc acgcgtggt

19

<210> 9

<211> 26

<212> DNA

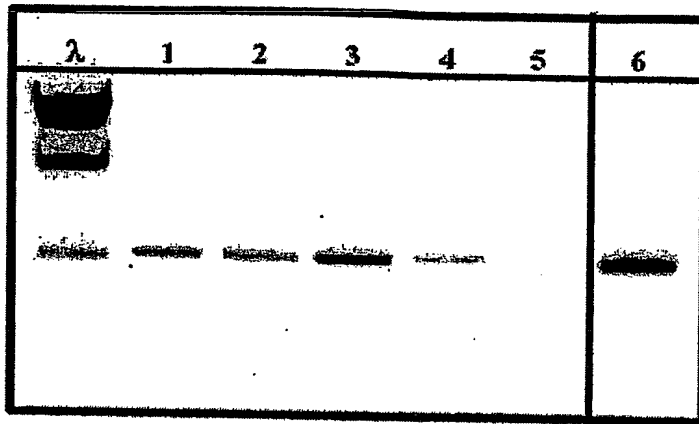
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer

<400> 9

agacggcgcc ggagaggacg aagcga



**Figure 1. RT-PCR analysis of barley limit dextrinase inhibitor expression.**

**Figure 2. Northern blot analysis of limit dextrinase expression.**

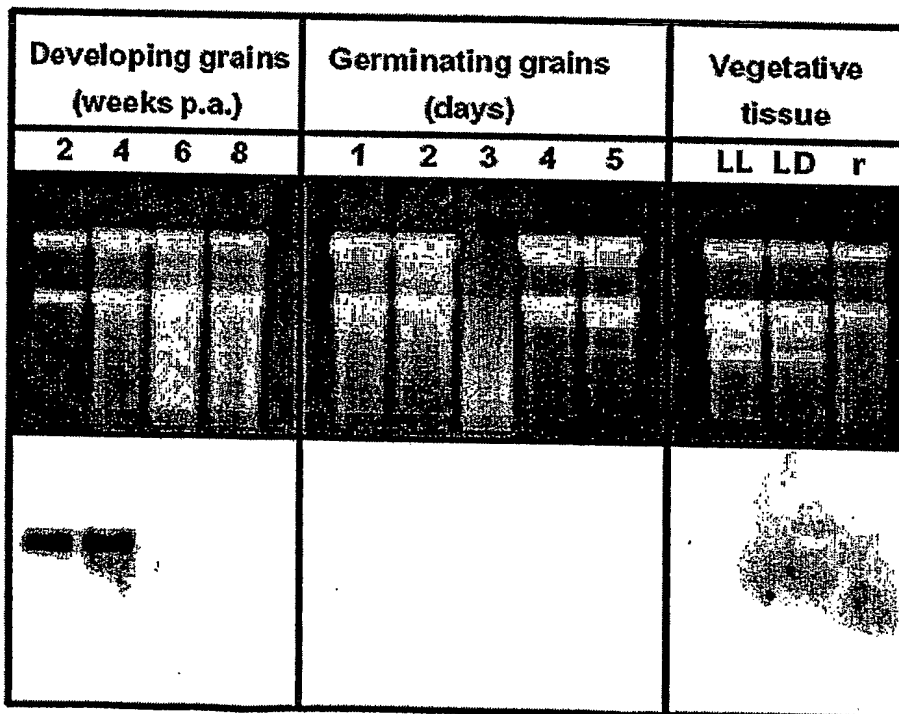


Figure 3. Southern blot analysis of limit dextrinase inhibitor in barley genomic DNA.

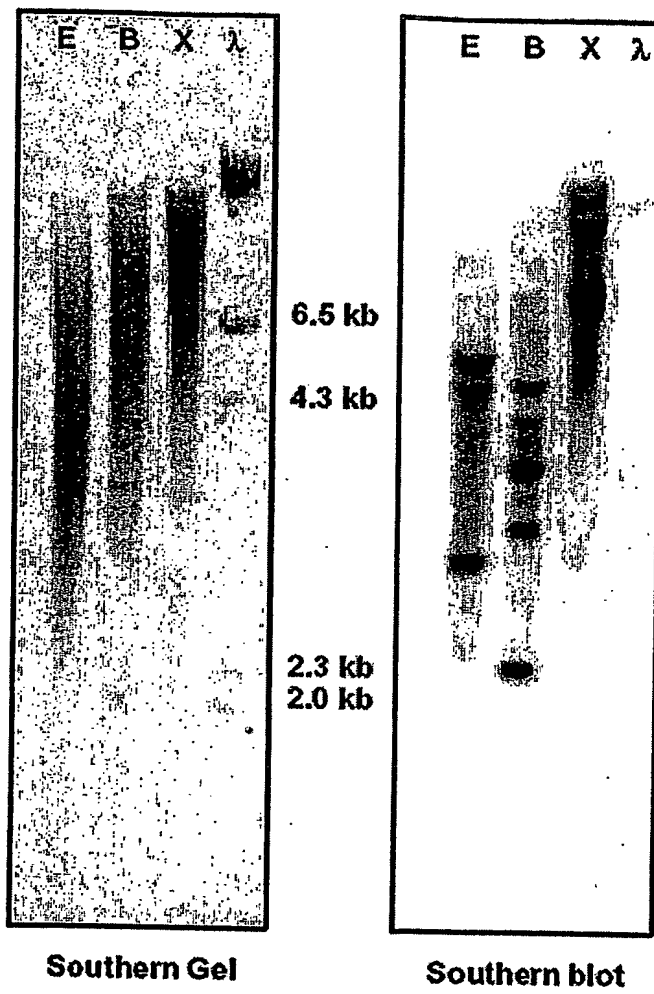


Figure 4. Isolation of limit dextrinase inhibitor promoter fragments by genome walking PCR.

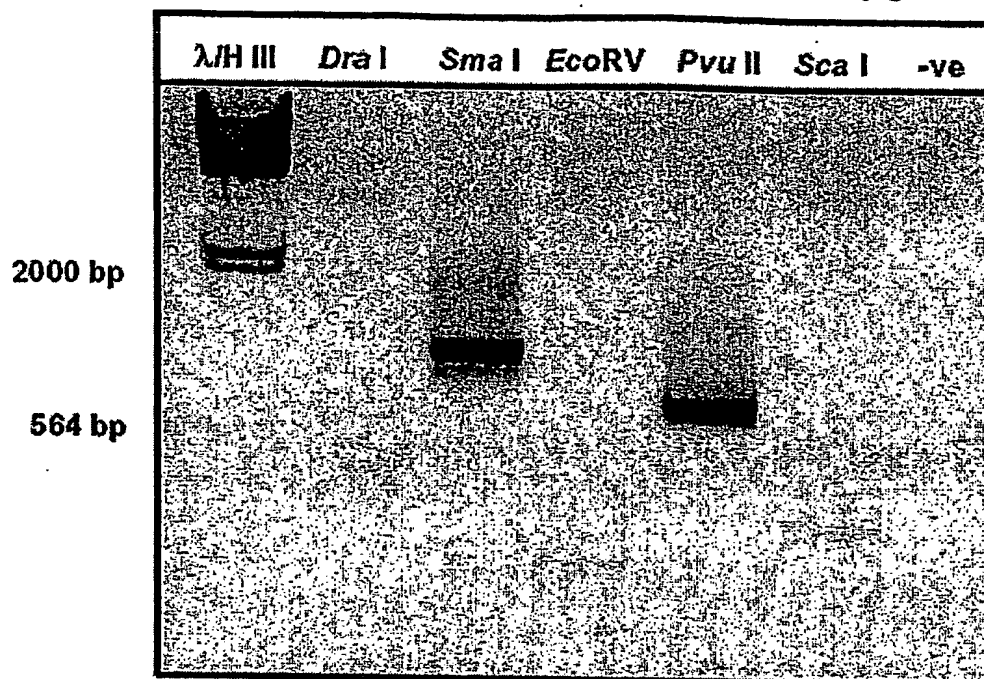


Figure 5. Barley transformation construct containing the limit dextrinase inhibitor promoter.

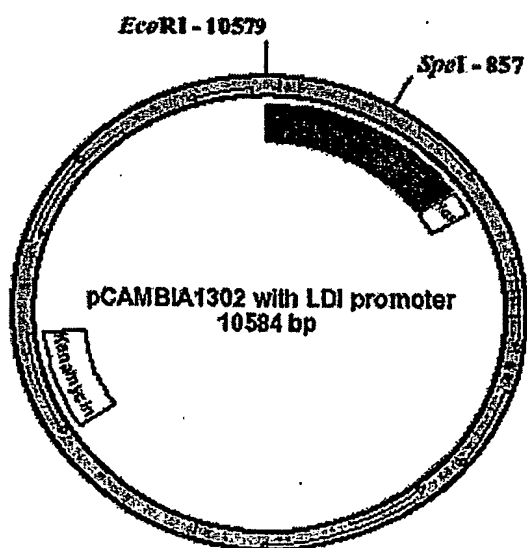


Figure 6. Transient expression assays using the limit dextrinase promoter: GFP construct.

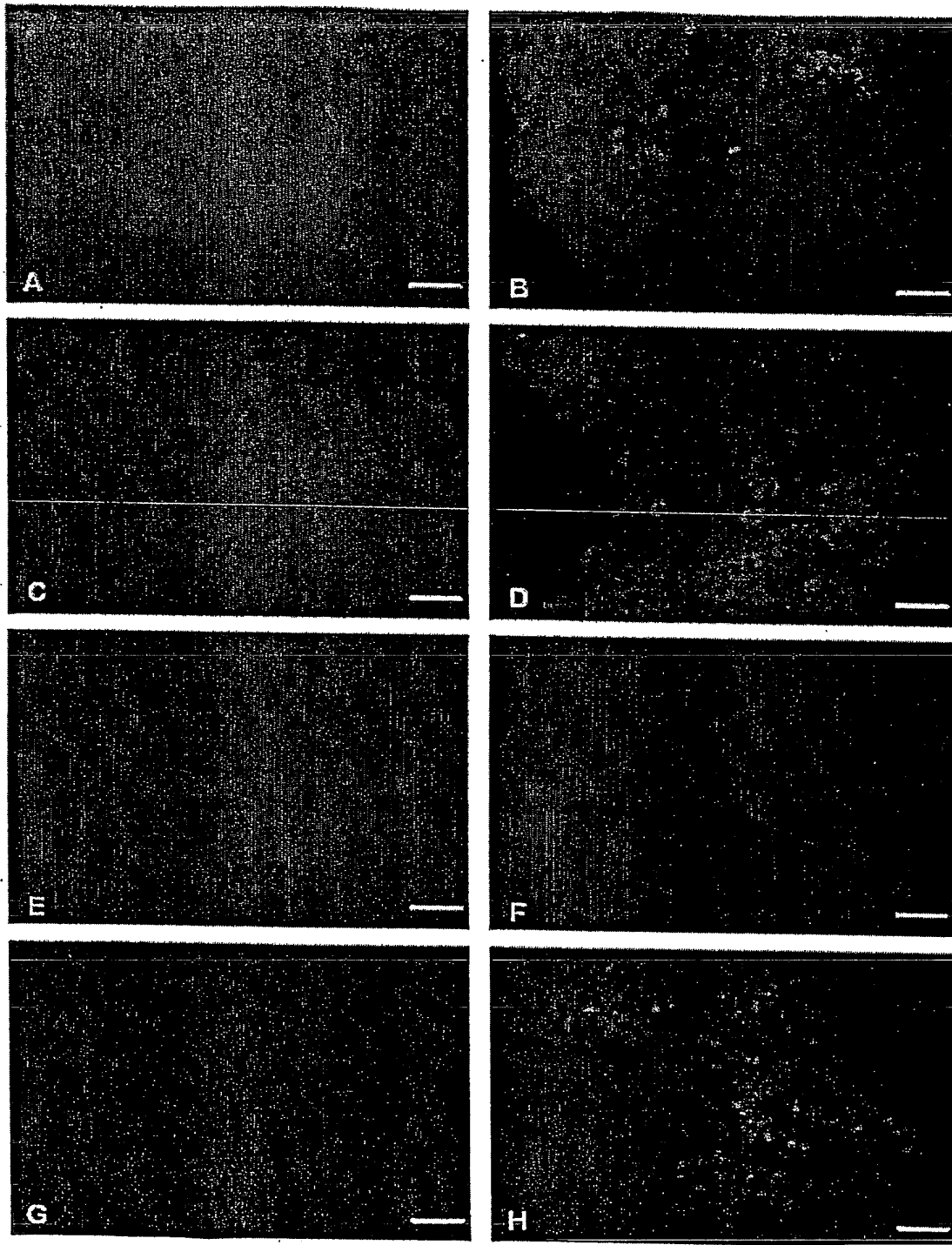


Figure 7.

Start	End	Score	Promoter Sequence
247	297	0.89	ATGCCCCGTGTAAAAGAAACCCCTTCTTTCCTAAAAATATA <sup>A</sup> TATCATCGT
273	323	0.95	TTTCCTAAAAATATATATCATCGTCCGTCATGATACGTTT <sup>A</sup> TGTATTCAA
652	702	0.83	CAAGAACCTCCAAATAAACGCCAACAAGAAAGAAATGAGC <sup>A</sup> TTACTTGCG

Figure 8

Sequences producing significant alignments:			Score (bits)	E Value
gi 18868 emb X13443.1 HVAATI	Barley mRNA for alpha-amylase/...		82	2e-12
gi 22035404 gb AY124482.1	Danio rerio myogenin gene, exons...		80	6e-12
gi 24614584 gb AY050653.1	Giardia intestinalis SEC24 (SEC2...		76	1e-10
gi 10938038 gb AF162890.1 AF162890S1	Mus musculus peroxisom...		76	1e-10
gi 5821237 dbj AB021922.1	Homo sapiens gene for lectin-lik...		76	1e-10
gi 3721562 dbj AB011276.1	Mus musculus gene for alphala ca...		74	4e-10
gi 30313388 gb AY099112.1	Rattus norvegicus obese protein ...		72	2e-09
gi 18140057 gb AF457660.1	Castanea dentata clone ACS2 vasc...		72	2e-09
gi 4104807 gb AF039526.1 AF039526	Homo sapiens MHC class I ...		72	2e-09
gi 8050595 gb AF233737.1 AF233737	Agrotis ipsilon AiC5 chym...		72	2e-09
gi 6690643 gb AF191544.1 AF191544	Homo sapiens estrogen rec...		72	2e-09
gi 6164589 gb AF051769.1 AF051769	Homo sapiens hyaluronidas...		72	2e-09
gi 3643823 gb AF075270.1 AF075270	Hordeum vulgare high affi...		72	2e-09
gi 19919401 gb AF435445.1	Pleurotus ostreatus manganese pe...		70	6e-09
gi 8132114 gb AF153014.1	Trichomonas vaginalis Tvp14 (tvp1...		70	6e-09
gi 4456992 gb AF077743.1 AF077743	Mus musculus transcriptio...		70	6e-09
gi 12276207 gb AF269146.1 AF269146	Bilophila wadsworthia ta...		70	6e-09
gi 14275833 emb AJ289605.1 MMU289605	Mus musculus partial L...		70	6e-09
gi 3378604 emb AJ009889.1 HSAJ9889	Homo sapiens GHR gene, V...		70	6e-09
gi 3916231 gb AF074397.1 AF074397	Homo sapiens anti-mulleri...		70	6e-09
gi 5139506 emb Z18892.2 MMDESMINP	Mus musculus desmin gene		70	6e-09
gi 15487305 dbj AB060299.1	Mus musculus gene for acetyl Co...		70	6e-09
gi 12697590 dbj AB046716.1	Homo sapiens hST3Gal I gene for...		70	6e-09
gi 25453365 gb AY050651.2	Giardia intestinalis MYB (MYB) m...		68	2e-08
gi 13183059 gb AF237414.1	Anaplasma phagocytophilum RNA po...		68	2e-08
gi 13625520 gb AY014277.1	Lolium perenne gibberellin 20-ox...		68	2e-08
gi 17105179 gb AF439558.1 AF439558	Mus musculus X2CR1 gene,...		68	2e-08
gi 16209547 gb AY052528.1	Glycine max calmodulin isoform-4...		68	2e-08
gi 15213480 gb AF241535.1 AF241535	Homo sapiens mucin 4 (MU...		68	2e-08
gi 15558849 emb AJ310936.1 FSO310936	Fusarium solani chy ge...		68	2e-08
gi 4878023 gb AF131239.2 AF131239	Rattus norvegicus alpha 1...		68	2e-08
gi 15216031 emb AJ318812.1 VFA318812	Vicia faba var. minor ...		68	2e-08
gi 4454294 emb AJ132779.1 MMU132779	Mus musculus p107 gene ...		68	2e-08
gi 3133088 emb Y16736.1 HSA16736	Homo sapiens dif-2 gene, p...		68	2e-08
gi 6690521 gb AF154245.1 AF154245	Rattus norvegicus chemota...		68	2e-08
gi 5731977 gb AF114032.1 AF114032	Mus musculus glycogenin-1...		68	2e-08
gi 26453412 dbj AB094665.1	Seriola quinqueradiata YGHL1 ge...		68	2e-08
gi 4039145 gb AF099083.1 AF099083	Homo sapiens growth hormo...		68	2e-08
gi 2739123 gb AF029214.1 MMOX2S1	Mus musculus MRC OX-2 anti...		68	2e-08
gi 2895903 gb AF046916.1 AF046916	Ruminococcus flavefaciens...		68	2e-08
gi 1916583 gb U53907.1 RNU53907	Rattus norvegicus microsate...		68	2e-08



7/21

gi 6683556 dbj AB024534.1	Rattus norvegicus gene for thiaz...	68	2e-08
gi 7109286 gb AF227508.1	Rattus norvegicus intestinal alka...	66	1e-07
gi 13345792 gb AF332759.1	Homo sapiens partially duplicate...	66	1e-07
gi 5002511 emb Z98266.1 HSZ98266	Homo sapiens gene encoding...	66	1e-07
gi 6560627 gb AF112228.1 HSCD30P1	Homo sapiens CD30 protein...	66	1e-07
gi 24475537 dbj AB084484.1	Betula platyphylla DNA, microsa...	66	1e-07
gi 28875405 gb AF515463.1	Biomphalaria glabrata fibrinogen...	64	4e-07
gi 18140058 gb AF457661.1	Castanea dentata clone ACS10A va...	64	4e-07
gi 2581766 gb U77633.1 RNU77633	Rattus norvegicus chromosom...	64	4e-07
gi 2764739 emb AJ002743.1 HSAJ2743	Homo sapiens cardiophroph...	64	4e-07
gi 7579914 emb AJ277249.1 HSA277249	Homo sapiens partial HR...	64	4e-07
gi 1518845 gb U63899.1 MMU63899	Mus musculus Girk2 gene, pr...	64	4e-07
gi 7109284 gb AF227507.1	Rattus norvegicus intestinal alka...	62	2e-06
gi 12744733 gb AF318503.1	Danio rerio Myod (myod) gene, co...	60	6e-06
gi 2581767 gb U77634.1 RNU77634	Rattus norvegicus chromosom...	60	6e-06
gi 14251200 gb AF220499.2 AF220499	Acidithiobacillus ferroo...	60	6e-06
gi 14043019 gb AF221946.2 AF221946	Rickettsia rickettsii ce...	60	6e-06
gi 2342636 emb Y11638.1 MMY11638	M.musculus CYP4A14 gene, e...	60	6e-06
gi 18873678 emb AJ272507.1 HSA272507	Homo sapiens partial K...	60	6e-06
gi 2564335 dbj AB008218.1 AB008218S1	Homo sapiens gene for ...	60	6e-06
gi 17907575 emb AJ409277.1 CDR409277	Camelus dromedarius pa...	58	2e-05
gi 5091690 gb AF139181.1 AF139181	Bartonella henselae S-ade...	58	2e-05
gi 14164368 dbj AB052355.1 AB05234S16	Mus musculus gene for...	58	2e-05
gi 15081477 gb AF401090.1	Wolbachia pipientis RNA polymera...	56	9e-05
gi 17298240 gb AF283339.1 F283327S13	Homo sapiens candidate...	56	9e-05
gi 4139055 gb AF072833.1 AF072833	Homo sapiens SP23 gene, p...	56	9e-05
gi 17298258 gb AF283357.1 F283327S31	Homo sapiens candidate...	54	4e-04
gi 18413572 emb AJ428930.1 XLA428930	Xenopus laevis partial...	54	4e-04
gi 18873687 emb AJ272516.1 HSA272516	Homo sapiens partial K...	54	4e-04
gi 29648446 gb AY190007.1	Pan paniscus clone BoE26-M13R LI...	52	0.001
gi 22651882 gb AF291761.1	Ipomoea batatas S-adenosylmethio...	52	0.001
gi 22347797 gb AF532732.1	Danio rerio mx gene, promoter se...	52	0.001
gi 11230634 emb AJ289159.1 HSA289159	Homo sapiens CD30 gene...	52	0.001
gi 3342093 gb AF074905.1 HOMOSLC04	Homo sapiens neuronal an...	50	0.006
gi 13377504 gb AF325198.1 AF325198	Triticum aestivum LRR14 ...	48	0.023
gi 6649909 gb AF026274.1 AF026274	Mus musculus Sumiko (sumi...	48	0.023
gi 6224791 gb AF190816.1 AF190816	Homo sapiens complement f...	48	0.023
gi 4104439 gb AF035664.1 HSTGFRBI3	Homo sapiens transformin...	48	0.023
gi 21213045 emb AJ487974.1 EAM487974	Enterobacter amnigenus...	48	0.023
gi 15638904 gb AC024246.8	Homo sapiens BAC clone RP11-6430...	46	0.090
gi 15638822 gb AC079120.6	Homo sapiens BAC clone RP11-345M...	46	0.090
gi 13793999 gb AY029002.1	Pinus taeda isolate PTLTP3-iii27...	46	0.090
gi 11093799 gb AF285184.1 AF285184	Mus musculus basic trans...	46	0.090
gi 2984654 gb AF039088.1 AF039088	Homo sapiens non-hepatic ...	46	0.090
gi 3342092 gb AF074904.1 HOMOSLC03	Homo sapiens neuronal an...	44	0.36
gi 29029467 gb AY173030.1	Danio rerio zinc finger transcri...	44	0.36

8/21

gi 2281684 gb AF009433.1 AH006708S06	Homo sapiens clone 22 ...	<u>44</u>	0.36
gi 13936217 gb AY029021.1	Pinus radiata isolate PRLTP3-iii...	<u>44</u>	0.36
gi 13936206 gb AY029014.1	Pinus taeda isolate PTLTP9-iii15...	<u>44</u>	0.36
gi 13936204 gb AY029013.1	Pinus taeda isolate PTLTP4-i360 ...	<u>44</u>	0.36
gi 13794006 gb AY029007.1	Pinus taeda isolate PTLTP3-v94 n...	<u>44</u>	0.36
gi 13794003 gb AY029005.1	Pinus taeda isolate PTLTP6-i425 ...	<u>44</u>	0.36
gi 24210408 emb AJ320160.1 FNU320160	Fusobacterium nucleatu...	<u>44</u>	0.36
gi 13928026 emb AL121594.6 CNS01DRY	Human chromosome 14 DNA...	<u>44</u>	0.36
gi 8191116 gb AC040163.3 AC040163	Homo sapiens chromosome 1...	<u>44</u>	0.36
gi 6707080 gb AF139182.1 AF139182	Bartonella henselae filam...	<u>44</u>	0.36
gi 4028938 gb AC004230.1 AC004230	Homo sapiens Chromosome 1...	<u>44</u>	0.36
gi 21623971 dbj AP001094.6	Homo sapiens genomic DNA, chrom...	<u>44</u>	0.36
gi 19879812 dbj AP001363.4	Homo sapiens genomic DNA, chrom...	<u>44</u>	0.36

# Alignments

>gi|18868|emb|X13443.1|HVAATI Barley mRNA for alpha-amylase/trypsin inhibitor

Length = 672

Score = 81.8 bits (41), Expect = 2e-12

Identities = 41/41 (100%)

Strand = Plus / Plus

Query: 793 aagagattgaaccaacgaccaataaaactagtatcaacaatg 833

Sbjct: 1 aagagattgaaccaacgaccaataaaactagtatcaacaatg 41

>gi|22035404|gb|AY124482.1| Danio rerio myogenin gene, exons 1, 2 and 3 and complete cds

Length = 4260

Score = 79.8 bits (40), Expect = 6e-12

Identities = 40/40 (100%)

Strand = Plus / Minus

Query: 3 tcgattactatagggcacgcgtgggtcgacggccccgggctg 42

Sbjct: 4246 tcgattactatagggcacgcgtgggtcgacggccccgggctg 4207

>gi|24614584|gb|AY050653.1| Giardia intestinalis SEC24 (SEC24) mRNA, complete cds

Length = 1420

Score = 75.8 bits (38), Expect = 1e-10

Identities = 38/38 (100%)

Strand = Plus / Minus

Query: 5 gattactatagggcacgcgtgggtcgacggccccgggctg 42

Sbjct: 1417 gattactatagggcacgcgtgggtcgacggccccgggctg 1380

9/21

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 23 actatagggcacgcgtgggtcgacggcccgggctg 56

>gi|10938038|gb|AF162890.1|AF162890S1 Mus musculus peroxisomal assembly  
protein PEX3P (Pex3) gene,  
promoter and exon 1  
Length = 2785

Score = 75.8 bits (38), Expect = 1e-10  
Identities = 38/38 (100%)  
Strand = Plus / Plus

Query: 5 gattactatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 13 gattactatagggcacgcgtgggtcgacggcccgggctg 50

>gi|5821237|dbj|AB021922.1| Homo sapiens gene for lectin-like oxidized LDL  
receptor, promoter  
region  
Length = 2463

Score = 75.8 bits (38), Expect = 1e-10  
Identities = 38/38 (100%)  
Strand = Plus / Plus

Query: 5 gattactatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 13 gattactatagggcacgcgtgggtcgacggcccgggctg 50

>gi|3721562|dbj|AB011276.1| Mus musculus gene for alpha calcium  
channel, partial cds  
Length = 2099

Score = 73.8 bits (37), Expect = 4e-10  
Identities = 40/41 (97%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctgttattgg 49  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctgttattgg 41

10/21

>gi|30313388|gb|AY099112.1| Rattus norvegicus obese protein gene, 5'  
flanking region and  
partial cds  
Length = 1127

Score = 71.9 bits (36), Expect = 2e-09  
Identities = 36/36 (100%)  
Strand = Plus / Plus

Query: 7 ttactatagggcacgcgtgggtcgacggccccgggctg 42  
|||||  
Sbjct: 17 ttactatagggcacgcgtgggtcgacggccccgggctg 52

>gi|18140057|gb|AF457660.1| Castanea dentata clone ACS2 vascular protein  
gene, promoter  
region  
Length = 685

Score = 71.9 bits (36), Expect = 2e-09  
Identities = 36/36 (100%)  
Strand = Plus / Plus

Query: 7 ttactatagggcacgcgtgggtcgacggccccgggctg 42  
|||||  
Sbjct: 9 ttactatagggcacgcgtgggtcgacggccccgggctg 44

>gi|4104807|gb|AF039526.1|AF039526 Homo sapiens MHC class I related protein  
1 (MR1) gene, partial  
cds  
Length = 1423

Score = 71.9 bits (36), Expect = 2e-09  
Identities = 36/36 (100%)  
Strand = Plus / Plus

Query: 7 ttactatagggcacgcgtgggtcgacggccccgggctg 42  
|||||  
Sbjct: 4 ttactatagggcacgcgtgggtcgacggccccgggctg 39

>gi|8050595|gb|AF233737.1|AF233737 Agrotis ipsilon AiC5 chymotrypsinogen  
gene, promoter region  
Length = 951

Score = 71.9 bits (36), Expect = 2e-09  
Identities = 36/36 (100%)  
Strand = Plus / Plus

Query: 7 ttactatagggcacgcgtgggtcgacggccccgggctg 42  
|||||  
Sbjct: 11 ttactatagggcacgcgtgggtcgacggccccgggctg 46

11/21

>gi|6690643|gb|AF191544.1|AF191544 Homo sapiens estrogen receptor beta  
gene, promoter region and  
partial cds  
Length = 2495

Score = 71.9 bits (36), Expect = 2e-09  
Identities = 39/40 (97%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccggtgttattg 48  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccggtgttattg 40

>gi|6164589|gb|AF051769.1|AF051769 Homo sapiens hyaluronidase-like  
pseudogene 1 (HYALP1), partial sequence  
Length = 11957

Score = 71.9 bits (36), Expect = 2e-09  
Identities = 36/36 (100%)  
Strand = Plus / Minus

Query: 7 ttactatagggcacgcgtgggtcgacggcccggtg 42  
|||||  
Sbjct: 11954 ttactatagggcacgcgtgggtcgacggcccggtg 11919

>gi|3643823|gb|AF075270.1|AF075270 Hordeum vulgare high affinity sulfate  
transporter (HVST1) gene,  
promoter region, 5'UTR, and partial cds  
Length = 1094

Score = 71.9 bits (36), Expect = 2e-09  
Identities = 36/36 (100%)  
Strand = Plus / Plus

Query: 7 ttactatagggcacgcgtgggtcgacggcccggtg 42  
|||||  
Sbjct: 64 ttactatagggcacgcgtgggtcgacggcccggtg 99

>gi|19919401|gb|AF435445.1| Pleurotus ostreatus manganese peroxidase (mnp3)  
gene, promoter  
region and partial cds  
Length = 2790

Score = 69.9 bits (35), Expect = 6e-09  
Identities = 35/35 (100%)  
Strand = Plus / Plus

Query: 8 tactatagggcacgcgtgggtcgacggcccggtg 42  
|||||  
Sbjct: 79 tactatagggcacgcgtgggtcgacggcccggtg 113

12/21

>gi|8132114|gb|AF153014.1| Trichomonas vaginalis Tvp14 (tvp14) gene,  
complete cds

Length = 1463

Score = 69.9 bits (35), Expect = 6e-09

Identities = 35/35 (100%)

Strand = Plus / Plus

Query: 8 tactatagggcacgcgtgggtcgacggcccgggctg 42

|||||

Sbjct: 11 tactatagggcacgcgtgggtcgacggcccgggctg 45

>gi|4456992|gb|AF077743.1|AF077743 Mus musculus transcription factor TFEC  
gene, promoter region and

5' UTR

Length = 615

Score = 69.9 bits (35), Expect = 6e-09

Identities = 38/39 (97%)

Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctgttatt 47

|||||

Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctgttatt 39

>gi|12276207|gb|AF269146.1|AF269146 Bilophila wadsworthia taurine:pyruvate  
aminotransferase gene,

complete cds

Length = 2050

Score = 69.9 bits (35), Expect = 6e-09

Identities = 35/35 (100%)

Strand = Plus / Minus

Query: 8 tactatagggcacgcgtgggtcgacggcccgggctg 42

|||||

Sbjct: 2050 tactatagggcacgcgtgggtcgacggcccgggctg 2016

>gi|14275833|emb|AJ289605.1|MMU289605 Mus musculus partial Lancl1 gene  
for LanC-like protein 1, exon 4

Length = 682

Score = 69.9 bits (35), Expect = 6e-09

Identities = 35/35 (100%)

Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctgt 43

|||||

Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctgt 35

Score = 67.9 bits (34), Expect = 2e-08  
 Identities = 34/34 (100%)  
 Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccggtg 42  
 |||||  
 Sbjct: 46 actatagggcacgcgtgggtcgacggcccggtg 79

>gi|3378604|emb|AJ009889.1|HSAJ9889 Homo sapiens GHR gene, V1 promoter  
 region  
 Length = 1640

Score = 69.9 bits (35), Expect = 6e-09  
 Identities = 38/39 (97%)  
 Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccggtgttatt 47  
 |||||  
 Sbjct: 1 actatagggcacgcgtgggtcgacggcccggtgttatt 39

>gi|3916231|gb|AF074397.1|AF074397 Homo sapiens anti-mullerian hormone type  
 II receptor (AMHR2)  
 gene, promoter region and partial cds  
 Length = 1135

Score = 69.9 bits (35), Expect = 6e-09  
 Identities = 38/39 (97%)  
 Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccggtgttatt 47  
 |||||  
 Sbjct: 13 actatagggcacgcgtgggtcgacggcccggtgttatt 51

>gi|5139506|emb|Z18892.2|MMDESMINP Mus musculus desmin gene  
 Length = 19391

Score = 69.9 bits (35), Expect = 6e-09  
 Identities = 35/35 (100%)  
 Strand = Plus / Minus

Query: 8 tactatagggcacgcgtgggtcgacggcccggtg 42  
 |||||  
 Sbjct: 19391 tactatagggcacgcgtgggtcgacggcccggtg 19357

14/21

>gi|15487305|dbj|AB060299.1| Mus musculus gene for acetyl CoA  
synthetase-1, promoter region,  
partial sequence  
Length = 2094

Score = 69.9 bits (35), Expect = 6e-09  
Identities = 38/39 (97%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtggtcgacggccccgggctgttatt 47  
|||||  
Sbjct: 1 actatagggcacgcgtggtcgacggccccgggctgttatt 39

>gi|12697590|dbj|AB046716.1| Homo sapiens hST3Gal I gene for alpha 2,3-  
sialyltransferase I,  
promoter region  
Length = 1950

Score = 69.9 bits (35), Expect = 6e-09  
Identities = 38/39 (97%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtggtcgacggccccgggctgttatt 47  
|||||  
Sbjct: 1 actatagggcacgcgtggtcgacggccccgggctgttatt 39

>gi|25453365|gb|AY050651.2| Giardia intestinalis MYB (MYB) mRNA, complete  
cds  
Length = 3069

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Minus

Query: 9 actatagggcacgcgtggtcgacggccccgggctg 42  
|||||  
Sbjct: 3069 actatagggcacgcgtggtcgacggccccgggctg 3036

Score = 52.0 bits (26), Expect = 0.001  
Identities = 26/26 (100%)  
Strand = Plus / Plus

Query: 17 gcacgcgtggtcgacggccccgggctg 42  
|||||  
Sbjct: 1 gcacgcgtggtcgacggccccgggctg 26



15/21

>gi|13183059|gb|AF237414.1| Anaplasma phagocytophilum RNA polymerase beta subunit (rpoB) gene,  
complete cds  
Length = 4185

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Minus

Query: 9 actatagggcacgcgtgggtcgacggccccgggctg 42  
|||||  
Sbjct: 4041 actatagggcacgcgtgggtcgacggccccgggctg 4008

>gi|13625520|gb|AY014277.1| Lolium perenne gibberellin 20-oxidase gene,  
complete cds  
Length = 2128

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggccccgggctg 42  
|||||  
Sbjct: 15 actatagggcacgcgtgggtcgacggccccgggctg 48

>gi|17105179|gb|AF439558.1|AF439558 Mus musculus X2CR1 gene, promoter region and partial cds  
Length = 830

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggccccgggctg 42  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggccccgggctg 34

>gi|16209547|gb|AY052528.1| Glycine max calmodulin isoform-4 (cam-4) gene, promoter region  
and partial cds  
Length = 2050

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 5 gattactatagggcacgcgtgggtcgacggccccgg 38  
|||||  
Sbjct: 13 gattactatagggcacgcgtgggtcgacggccccgg 46

16/21

>gi|15213480|gb|AF241535.1|AF241535 Homo sapiens mucin 4 (MUC4) gene,  
promoter sequence and partial  
cds  
Length = 3716

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctg 34

>gi|15558849|emb|AJ310936.1|FSO310936 Fusarium solani chy gene for putative  
cyanide hydratase enzyme  
Length = 1981

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Minus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1981 actatagggcacgcgtgggtcgacggcccgggctg 1948

>gi|4878023|gb|AF131239.2|AF131239 Rattus norvegicus alpha 1,2-  
fucosyltransferase C (FTC) gene, complete  
cds  
Length = 1555

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Minus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1534 actatagggcacgcgtgggtcgacggcccgggctg 1501

>gi|15216031|emb|AJ318812.1|VFA318812 Vicia faba var. minor aap1 gene,  
promoter region  
Length = 1702

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctg 34

17/21

>gi|4454294|emb|AJ132779.1|MMU132779 Mus musculus p107 gene promoter region  
Length = 776

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctg 34

>gi|3133088|emb|Y16736.1|HSA16736 Homo sapiens dif-2 gene, promoter region  
Length = 1368

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 11 actatagggcacgcgtgggtcgacggcccgggctg 44

>gi|6690521|gb|AF154245.1|AF154245 Rattus norvegicus chemotactic protein-3  
gene, complete cds  
Length = 2416

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctg 34

>gi|5731977|gb|AF114032.1|AF114032 Mus musculus glycogenin-1 gene,  
promoter and partial cds  
Length = 1958

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctg 34

>gi|26453412|dbj|AB094665.1| Seriola quinqueradiata YGHL1 gene for putative  
growth hormone  
like protein-1, complete cds  
Length = 6658

18/21

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 39 actatagggcacgcgtgggtcgacggcccgggctg 72

>gi|4039145|gb|AF099083.1|AF099083 Homo sapiens growth hormone secretagogue  
receptor gene, 5'  
flanking region and partial cds  
Length = 1237

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctg 34

>gi|2739123|gb|AF029214.1|MMOX2S1 Mus musculus MRC OX-2 antigen homolog  
gene, exon 1  
Length = 2791

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Minus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 2791 actatagggcacgcgtgggtcgacggcccgggctg 2758

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggcccgggctg 42  
|||||  
Sbjct: 1 actatagggcacgcgtgggtcgacggcccgggctg 34

>gi|2895903|gb|AF046916.1|AF046916 Ruminococcus flavefaciens FD-1 glutamine  
synthetase type III (glnA)  
gene, complete cds  
Length = 2685

Score = 67.9 bits (34), Expect = 2e-08  
Identities = 34/34 (100%)  
Strand = Plus / Minus

Query: 9 actatagggcacgcgtgggtcgacggccccgggctg 42  
 |||||  
 Sbjct: 2685 actatagggcacgcgtgggtcgacggccccgggctg 2652

Score = 58.0 bits (29), Expect = 2e-05  
 Identities = 29/29 (100%)  
 Strand = Plus / Plus

Query: 14 agggcacgcgtgggtcgacggccccgggctg 42  
 |||||  
 Sbjct: 1 agggcacgcgtgggtcgacggccccgggctg 29

>gi|1916583|gb|U53907.1|RNU53907 Rattus norvegicus microsatellite sequence  
 D10Mco29

Length = 498

Score = 67.9 bits (34), Expect = 2e-08  
 Identities = 34/34 (100%)  
 Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggccccgggctg 42  
 |||||  
 Sbjct: 5 actatagggcacgcgtgggtcgacggccccgggctg 38

>gi|6683556|dbj|AB024534.1| Rattus norvegicus gene for thiazide-sensitive  
 Na-Cl

cotransporter, 5' flanking region  
 Length = 2145

Score = 67.9 bits (34), Expect = 2e-08  
 Identities = 34/34 (100%)  
 Strand = Plus / Plus

Query: 9 actatagggcacgcgtgggtcgacggccccgggctg 42  
 |||||  
 Sbjct: 1 actatagggcacgcgtgggtcgacggccccgggctg 34

>gi|7109286|gb|AF227508.1| Rattus norvegicus intestinal alkaline  
 phosphatase-II (IAP-II) gene,  
 complete cds  
 Length = 6359

Score = 65.9 bits (33), Expect = 1e-07  
 Identities = 33/33 (100%)  
 Strand = Plus / Minus

20/21

Query: 7     ttactatagggcacgcgtgggtcgacggcccggg 39  
             |||||  
Sbjct: 6349 ttactatagggcacgcgtgggtcgacggcccggg 6317

>gi|13345792|gb|AF332759.1|     Homo sapiens partially duplicated CHRNA7  
gene, hybrid intron A/4  
             and partial exon 5  
             Length = 1280

Score = 65.9 bits (33), Expect = 1e-07  
Identities = 36/37 (97%)  
Strand = Plus / Plus

Query: 11 tatagggcacgcgtgggtcgacggcccgggctgttatt 47  
             |||||  
Sbjct: 8     tatagggcacgcgtgggtcgacggcccgggctgttatt 44

>gi|5002511|emb|Z98266.1|HSZ98266     Homo sapiens gene encoding plakophilin  
(exons 1-13)  
             Length = 49999

Score = 65.9 bits (33), Expect = 1e-07  
Identities = 33/33 (100%)  
Strand = Plus / Plus

Query: 10 ctatagggcacgcgtgggtcgacggcccgggctg 42  
             |||||  
Sbjct: 16 ctatagggcacgcgtgggtcgacggcccgggctg 48

>gi|6560627|gb|AF112228.1|HSCD30P1     Homo sapiens CD30 protein (CD30) gene,  
promoter, partial sequence  
             Length = 1605

Score = 65.9 bits (33), Expect = 1e-07  
Identities = 37/39 (94%)  
Strand = Plus / Plus

Query: 9     actatagggcacgcgtgggtcgacggcccgggctgttatt 47  
             |||||  
Sbjct: 1     actatagggcacgcgtgggtcgacggcccgggctgttatt 39

>gi|24475537|dbj|AB084484.1|     Betula platyphylla DNA, microsatellite:BpA  
             Length = 427

Score = 65.9 bits (33), Expect = 1e-07  
Identities = 33/33 (100%)  
Strand = Plus / Minus

21/21

Query: 10 ctatagggcacgcgtggtcgacggccccgggctg 42  
|||||  
Sbjct: 317 ctatagggcacgcgtggtcgacggccccgggctg 285

>gi|28875405|gb|AF515463.1| Biomphalaria glabrata fibrinogen related  
protein 12.1 precursor  
(FREP12.1) gene, partial cds  
Length = 609

Score = 63.9 bits (32), Expect = 4e-07  
Identities = 32/32 (100%)  
Strand = Plus / Plus

Query: 11 tatagggcacgcgtggtcgacggccccgggctg 42  
|||||  
Sbjct: 1 tatagggcacgcgtggtcgacggccccgggctg 32

>gi|18140058|gb|AF457661.1| Castanea dentata clone ACS10A vascular protein  
gene, promoter  
region  
Length = 1157

Score = 63.9 bits (32), Expect = 4e-07  
Identities = 35/36 (97%)  
Strand = Plus / Plus

Query: 7 ttactatagggcacgcgtggtcgacggccccgggctg 42  
|||||  
Sbjct: 10 ttactatagggcacgcgtggtcgccggccccgggctg 45

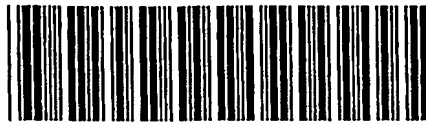
>gi|2581766|gb|U77633.1|RNU77633 Rattus norvegicus chromosome 10  
microsatellite D10Mco34  
Length = 1023

Score = 63.9 bits (32), Expect = 4e-07  
Identities = 33/34 (97%)  
Strand = Plus / Minus

Query: 9 actatagggcacgcgtggtcgacggccccgggctg 42  
|||||  
Sbjct: 1023 actatagggcacgcvtggtcgacggccccgggctg 990

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,  
or phase 0, 1 or 2 HTGS sequences)

PCT/GB2004/002589



THE PATENT OFFICE  
01 JUL 2004  
Received in, Received  
International Unit



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**